PB81-126583

Micromethods for Toxic Residue Screening by Negative Chemical Ionization Mass Spectrometry

Florida State Univ.
Tallahassee

Prepared for

Environmental Reseach Lab.-Duluth, MN

1980

U.S. Department of Commerce
National Technical Information Service
NTIS.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/J-80-086	2. Journal Article
3. REC	PB81-126583
4. TITLE AND SUBTITLE: Micromethods for Toxic Residue Screening by Negative Chemical Ionization Mass Spectrometry	5. REPORT DATE
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): Douglas W. Kuehl, Michael J. Whitaker and Ralph C. Dougherty	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: SAME	10. PROGRAM ELEMENT NO. A2NN1E
	11. CONTRACT/GRANT NO. Grant R8063340010
12. SPONSORING AGENCY NAME AND ADDRESS: U.S. Environmental Protection Agency, Environmental Research Laboratory, Office of Research and Development, Gulf Breeze, Fl 32561	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA/600/04

15. SUPPLEMENTARY NOTES: Ref: Analytical Chemistry 1980 52:935-940

16. ABSTRACT

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NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Toxicology Biochemistry Chromatography	Polychlorinated Chemical Residues Chlorophenols Chlorobiphenyls Negative Chemical Ionization Analysis Mass Spectrometry	06/A 06/T

18. DISTRIBUTION STATEMENT: Release to public	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 6
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

600/J-80-086
Journal Article

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Micromethods for Toxic Residue Screening by Negative Chemical Ionization Mass Spectrometry

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Methods were developed for the analysis of polychlorinated chemical residues found in milligram quantities of biological samples. Sample preparation by micro continuous liquid–liquid extraction steam distillation or by micro gel-permeation chromatography gave sufficiently clean residue extracts for negative chemical ionization analysis. With these techniques, chemicals such as chlorophenols and chlorobiphenyls have been confirmed in human adipose samples as small as 12.5 mg. These methods make it possible to screen <1-g samples of biological substrates for contamination with persistent toxic substances at part-per-billion levels.

Analytical procedures developed for the analysis of persistent toxic substances in environmental samples generally require between 10–500 g of substrate. However, increasing concern for the impact of environmental contamination on human health has stimulated the development of microsample methodology for residue analysis. The objective of this work was the development of an analytical procedure for the isolation and identification of persistent toxic substances at the parts-per-million to parts-per-billion level in <1-g samples of biological fluids. Our analytical strategy involves the use of efficient but simple separation techniques for sample cleanup followed by screening the cleaned up extracts with direct probe negative chemical ionization (NCI) mass spectrometry (*1*, *2*).

The two clean-up methodologies have focused on gel permeation chromatography (*3*–*7*) and steam distillation with continuous liquid–liquid extractions (*8*). Both of these techniques effectively separate persistent toxic substances from the bulk of the biomolecules, but do not involve adsorption chromatography which may reduce recoveries of the more polar chemicals.

Negative chemical ionization is uniquely suited to screening micro quantities of environmental samples for toxic chemicals, especially polychlorinated chemicals, because of high sensitivity for these chemicals and a virtual transparency to potentially interfering biomolecules. The ion forming reactions that are important for polychlorinated molecules are:

resonance capture of a thermal electron

$$M + e^-_s \rightarrow M^{-\cdot *} \xrightarrow{N} M^{-\cdot} \quad (1)$$

disassociative capture of a thermal electron

$$A - B + e^-_s \rightarrow A^- + B \quad (2)$$

chloride attachment

$$M + X^- \rightarrow MX^- \quad (3)$$

deprotonation

$$A - H + B^- \rightarrow A^- + BH \quad (4)$$

oxygen exchange

$$A - Y^{-\cdot} + O_2 \rightarrow AO^- + YO \quad (5)$$

EXPERIMENTAL

Gel Permeation Chromatography. *System Description.* The GPC system is similar to that previously described (*7*) with the following modifications. A 500 mm × 10 mm glass column (Ace Glass Company) was packed with 200–400 mesh bio-beads SX-8 (Bio-Rad Inc.) and eluted with cyclohexane–methylene chloride (1:1) at 1.5 mL/min. A 254-nm ultraviolet detector (Varian Aerograph) and a strip chart recorder (Linear Inc., model 260) were used to monitor the effluent.

Recovery Studies. Arochlor (1254 (100 ng), pentachlorophenol (50 ng), and hexachlorobenzene (50 ng) were spiked into 100 mg

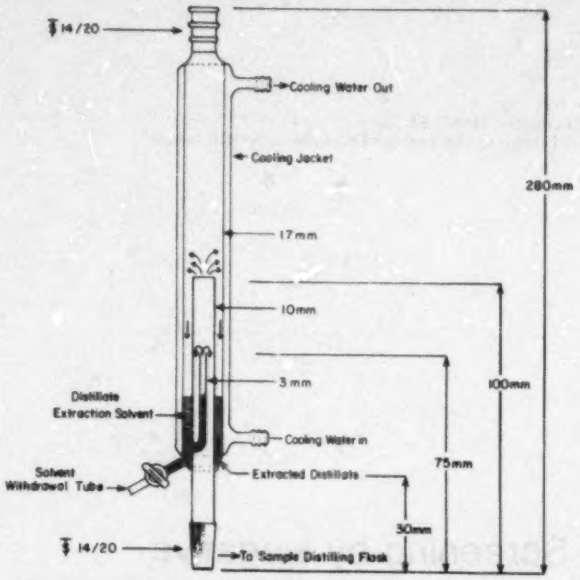

Figure 1. Microcontinuous liquid/liquid extraction steam distillation apparatus

of corn oil dissolved in 2.0 mL of methylene chloride and injected onto the gel-permeation chromatography (GPC) system. The 35–60 mL fractions were collected for analysis. Recoveries of these compounds were determined by electron capture gas chromatography (EC/GC). Pentachlorophenol was derivatized with Tri-Sil-BSA (Pierce Chemical Company) prior to GC analysis.

Sample Cleanup. In a typical experiment, 250 mg of human adipose tissue was mixed with an equal amount of anhydrous sodium sulfate. The mixture was extracted with 10 mL of hexane–acetone (1:1) in a tissue blender (Vertis). The solids were separated by centrifugation and decanting, and the solvent was reduced to 1.5 mL by concentration through a micro-Snyder column. The extract was injected into the GPC system and the 40–60 mL fraction was collected and concentrated through a micro-Snyder column for examination by NCI.

Procedural blanks were obtained by preparing a sample in the same manner without the tissue.

Steam Distillation. *System Description.* The steam distillation apparatus (Figure 1) was a modification of a similar unit previously described (*8*).

Recovery Studies. Anthracene, *trans*-chlordane, pentachlorophenol, and hexachlorobenzene in acetone were spiked into two 0.5-g samples of eastern oyster (*Crassostrea virginica*) tissue at 25 and 250-ng levels, and added to 35 mL of 10% H_2SO_4 in a 50-mL round bottom flask. The flask was placed in an ultrasonic bath for 20 min. The round bottom flask was then fitted onto the distillation head which had been loaded with 2 mL of water and 2 mL of 2,2,4-trimethylpentane. The flask was heated to 80 °C for 20 min, then to 100 °C for 40 min to distill the sample. The trimethylpentane was drained from the head and concentrated to 1.0 mL with a stream of dry nitrogen for GC determination of recoveries. Pentachlorophenol was derivatized with Tri-Sil-BSA (Pierce Chemical Company) prior to GC analysis. The chlorinated hydrocarbons were quantitated by EC/GC and the anthracene was quantitated by FID/GC.

The unspiked tissue served as a procedural blank.

Sample Cleanup. In a typical experiment 0.5 g of human adipose tissue was added to 35 mL of 10% H_2SO_4 in a 50-mL round bottom flask. The sample was then treated as described above. The drained trimethylpentane was concentrated to 100 mL with a stream of dry nitrogen for NCI analysis.

Procedural blanks were obtained by preparing a sample in the same manner without the tissue.

Reagents. Hexane, acetone, cyclohexane, methylene chloride, and 2,2,4-trimethylpentane were redistilled pesticide grade (Burdick and Jackson). Water was distilled-deionized and refluxed for 45 min on a steam distillation apparatus. Sulfuric acid (Baker Chemical Company) was diluted to 10% with the refluxed water. Standards for the recovery studies were obtained from the U.S. Environmental Protection Agency Repository at Research Triangle Park, N.C.

Gas Chromatography. A Varian Aerograph model 1400 EC/GC and a Varian Aerograph model 1700 FID/GC were equipped with identical 2 m × 2 mm glass columns packed with 3% OV-101 on gas Chrom Q. The EC/GC was operated isothermally at 185 °C and the FID/GC was temperature programmed from 100–200 °C at 4°/min. The carrier gas was N_2, 25 mL/min.

Mass Spectrometer. An AEI MS-902 mass spectrometer equipped with a chemical ionization source (Scientific Research Instruments Corporation) was operated in the negative ion mode with an electron energy of 470 eV, source temperature of 150 °C, source pressure of 0.5 Torr, and resolution 4000. A mixture of isobutane, methylene chloride, and oxygen (80:18:2) was used as the reagent gas. Direct probe sample introduction was used, and 8-s scans were recorded at 30, 60, 90, and 120 s after introduction on oscillographic paper.

The voltage of the electron multiplier (≈3.0 kV) was set such that the $(M + Cl)^-$ ion (m/z 441) for *trans*-chlordane, the $(M - 19)^-$ ion (m/z 357) for octachlorostyrene, and the $(M - 1)^-$ ion (m/z 263) for pentachlorophenol gave approximately a 50% scale reading on the oscillographic output, when spiked into a residue obtained from the steam distillation of 0.5 g of oyster.

RESULTS AND DISCUSSION

Several steam distillation units similar to the one described by Veith and Kiwus (*8*) were constructed and tested until a design was found that allowed the use of distillation volumes as small as 25 mL and also required minimal heat for steam distillation. The design that met these requirements is shown in Figure 1. Recoveries of hexachlorobenzene, pentachlorophenol, *trans*-chlordane, and anthracene spiked into 0.5 g of oyster tissue at 50 and 500 ppb ranged from 85% to 97%. These recoveries are comparable to those observed by Veith

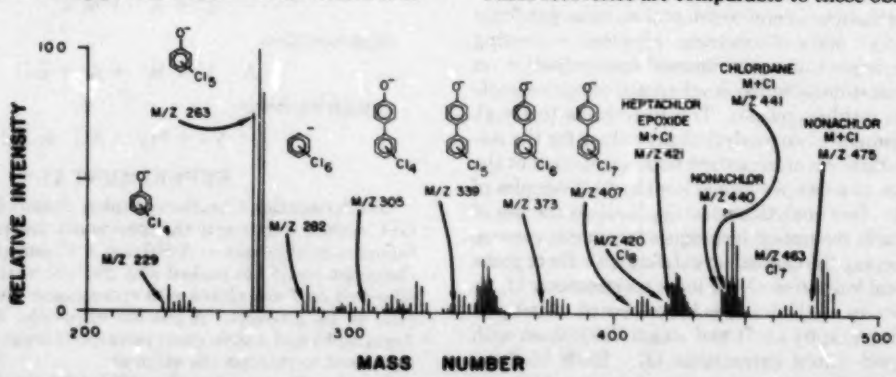

Figure 2. Typical NCI mass spectrum for steam distillation of 250 mg of human adipose tissue. Only ^{12}C negative mass defect ions are sho for polychlorinated chemicals

Table I. Polychloro Ion Clusters in NCI Spectra of Steam Distillates of 250-mg Samples of Human Adipose Tissue[a]

m/z	no. of chlorine	2-20-2-MW-1	2-20-1-MW-1	2-24-8-MW-1	2-24-7-MW-1	2-24-5-MW-1	3-35-6-MW-1	2-20-3-MW-1	2-20-4-MW-1	B-1[b]	identification
217	3	*									
231	4	*									
243	3	*									
263	5	*						*			pentachlorophenol (M – 1)⁻
294	4		*					*			
305	5	*	*	*	*	*	*	*	*		pentachlorobiphenyl (M – 19)⁻
307	4							*			
314	5		*								
317	4	*	*	*	*	*		*	*		p,p'-DDMU (M + 35)⁻
323	4							*			
339	5	*						*	*	*	hexachlorobiphenyl (M – 19)⁻
341	4		*		*	*	*	*	*		
350	4		*								
351	5							*			
373	6	*	*	*	*	*	*	*	*	*	p,p'-DDE (M + 35)⁻
375	5	*	*		*		*	*	*		heptachlorobiphenyl (M – 19)⁻
387	6							*			
407	7	*	*	*	*	*	*	*	*		p,p'-DDT (M – 35)⁻
409	6		*		*		*	*			octachlorobiphenyl (M – 19)
420	8		*	*	*	*	*	*			
421	7		*	*	*	*	*	*			
440	9		*	*	*	*	*	*			heptachlor epoxide (M– Cl)⁻
441	9		*	*	*	*	*	*	*		nonachlor M⁻·
463	7	*	*	*	*	*	*	*	*		chlordane (M – CL)⁻
475	10		*	*	*	*	*	*	*		nonachlor (M + CL)⁻

[a] An asterisk indicates the presence of the ion cluster at five times the noise or higher intensity. [b] Column labelled B-1 was the procedural blank. The ion clusters appearing in these spectra were at least an order of magnitude lower in intensity than in the sample spectra.

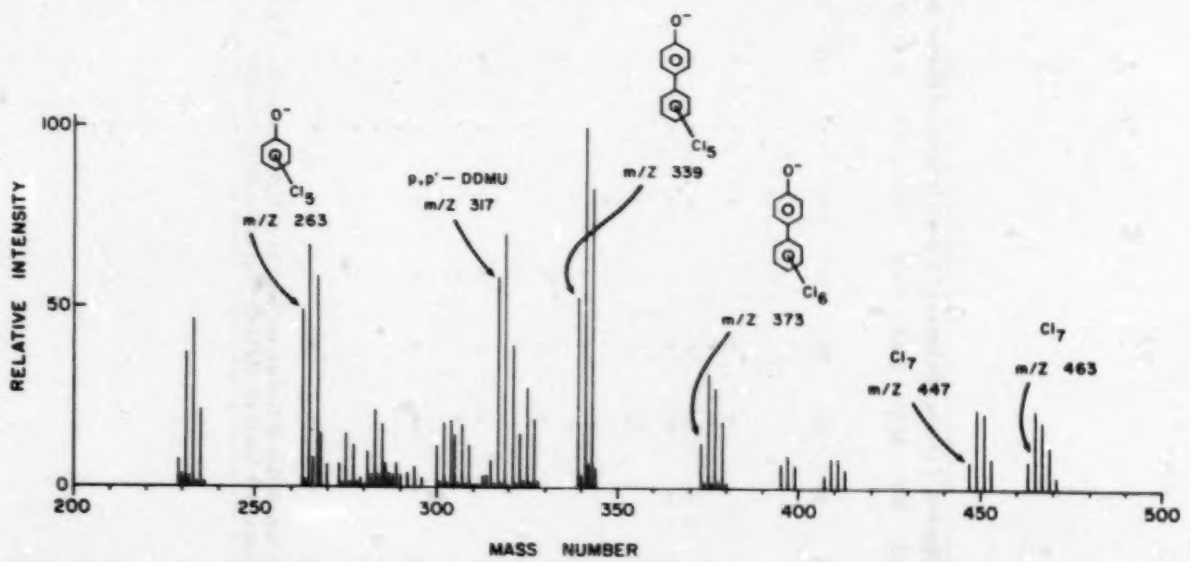

Figure 3. Typical NCI mass spectrum for steam distillation of 2.0 mL of human seminal plasma. Only ^{12}C negative mass defect ions are shown for polychlorinated chemicals

and Kiwus (8) for polychlorinated biphenyls (PCBs) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE) from fish tissue.

Two experiments were conducted to evaluate the utility of the microsteam distillation as a clean-up procedure for NCI screening for persistent toxic residues. First, we examined eight 250-mg samples for human adipose tissue and fourteen 2.0-mL samples of human seminal plasma to determine the different types of chemicals that may be isolated and identified in real samples by these techniques. Second, we analyzed smaller and smaller quantities of the same adipose tissue to determine the minimum quantity of tissue required for detection of the major toxic chemicals in these samples. The predominant contaminants identified in the adipose tissue survey were components of *tech*-chlordane, PCB isomers, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT) and 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDMU) (Table I). The predominant contaminants identified in human seminal plasma were PCB isomers and pentachlorophenol (Table II). The mass assignments in Tables I and II are pseudomolecular ions that were formed by reactions 1–5 above. Compound identifications are based upon a comparison with NCI spectra of standards. In each sample an ion at m/z 463 (Cl_7) was observed; however, this compound remains unidentified. Typical NCI mass spectra for steam distillation of 250 mg of adipose tissue and 2.0 mL of human seminal

Table II. Polychloro Ion Clusters in NCI Spectra of Steam Distillates of 2.0-mL Samples of Human Seminal Plasma[a,b]

sample no.		D-7	N-2	N-5	D-12	D-2	N-21	N-14	N-10	D-5	N-1	S-7	N-22	S-10	N-29	B-1	B-9	B-11	
sperm density (10^6/mL)		8	11	12	19	20	20	20	29	106	129	146	198	227	229	X	X	X	
m/z	no. of chlorine																		
212	3	*	*	*															
229	4			*			*	*											
231	4	*		*	*		*	*		*	*	*	*	*					compound identification
263	5	*		*		*	*	*							*				
285	3			*	*	*	*	*		*	*	*	*	*					
287	5				*					*	*	*	*	*	*				tetrachlorophenol (M – 1)⁻
301	4						*								*				
305	4		*	*															pentachlorophenol (M – 1)⁻
313	5									*									
317	4	*	*	*				*	*			*							
321	4	*		*			*	*	*	*		*		*					
323	6				*							*	*	*	*				pentachlorobiphenyl (M – 19)⁻
339	5		*				*												
343	3		*	*	*		*	*	*	*	*	*	*	*					*p,p'*-DDMU (M + CL)⁻
345	3			*			*	*	*										
373	6	*	*						*			*	*	*			*	*	
407	7		*	*	*	*	*	*											hexachlorobiphenyl (M – 19)⁻
427	7	*							*	*	*	*	*	*	*	*			
429	5										*								
447	5										*								heptachlorobiphenyl (M – 19)⁻
463	7	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	octachlorobiphenyl (M – 19)⁻

[a] An asterisk indicates observation of the ion cluster at five times the raise or higher intensity. [b] Columns labeled B-1, 9, 11 were procedural blanks. The ion clusters appearing in these spectra were at least an order of magnitude lower in intensity than in the sample spectra.

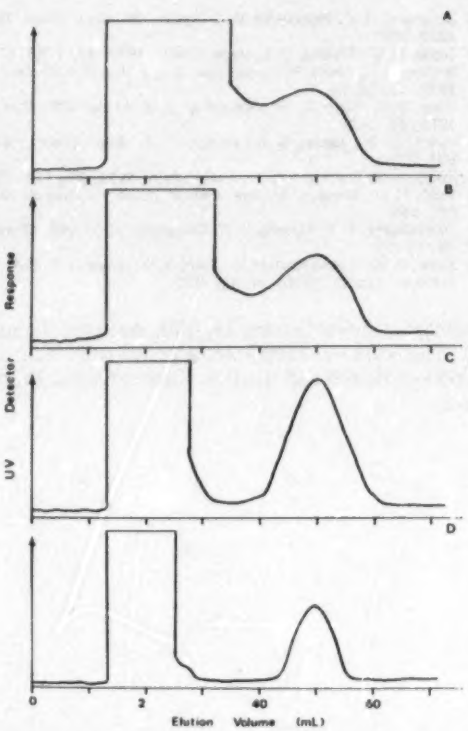

Figure 4. Gel-permeation chromatographic traces of UV detector response vs. elution volume for human adipose tissue samples of (A) 10 mg, (B) 50 mg, (C) 25 mg, and (D) 12.5 mg

plasma are shown in Figures 2 and 3, respectively. In addition to the toxic substances listed in Tables I and II, low molecular weight free fatty acids readily steam distill. Since free fatty acids have a high sensitivity for chloride attachment (*9*), they are the most consistent interference in NCI screening of environmental samples. Polychlorinated chemicals are therefore identified in the mass spectra by their negative mass defects as well as the characteristic isotopic ratios of the chlorine isotope clusters. In both the steam distillation and GPC procedures, a pentachlorobiphenyl was observed in the blanks at a level approximately an order of magnitude lower than that observed in the smallest samples. Pentachlorobiphenyl was therefore reported to be present only if it was 10 times greater than that observed in the blank.

Additional samples of 100, 50, and 25 mg of a human adipose tissue (2-20-2-MW-I, Table I) were analyzed. Although absolute peak intensity for identification decreased with decreasing sample size, all identifications made on the 250-mg sample were also possible on the 25-mg sample. An estimate of detectability of polychlorinated chemicals by NCI was established by observing the pseudomolecular ion of *trans*-chlordane, pentachlorophenol, and octachlorostyrene, which had been spiked into the residue of 500 mg of steam distilled cleaned up oyster tissue at 10 ppb. In each case the pseudomolecular ions were readily identifiable.

We have examined GPC as an alternate clean-up procedure. The system described by Kuehl and Leonard (*7*) was capable of isolating both polar and nonpolar toxic chemicals from fatty tissue; however, the system described was used for chromatography of 0.5–1.0 g of oil, which represented 5–10 g of a fish of 10% fat content. A new column 500 mm × 10 mm packed with Bio-Rad SX-8 beads and eluted at 1.5 mL/min with cyclohexane/methylenechloride provided separation of corn oil, PCBs, and pentachlorophenol comparable to that of the large columns (*7*). In addition, Arochlor 1254 (1 ppm), hexachlorobenzene (500 ppb), and pentachlorophenol (500 ppb) spiked into corn oil and eluted on this system were recovered at greater than 95%.

A series of 100-, 50-, 25-, and 12.5-mg samples of the same adipose tissue (2-12-2-MW-I, Table I) was then used to evaluate the GPC performance to determine if the same toxic chemicals could be found by GPC/NCI as were found by steam distillation/NCI. Figure 4 shows UV detector traces of the four tissue samples. As can be seen, maximum resolution and sensitivity occur with the 25-mg sample. Apparently a maximum optimum loading for this column was about 35 mg of oil. Negative chemical ionization analysis of each sample showed the same series of toxic compounds as was observed in the steam distillation/NCI experiment. In addition all compounds observed in the 100-mg sample were also observed in the 12.5-mg sample.

We applied the micro-GPC/NCI screening system to the analysis of a sample of Lake Ontario lake trout. A Soxhlet extract of 50 mg of trout tissue was cleaned up by GPC and analyzed by NCI. Polychlorinated biphenyls, hexachloro-

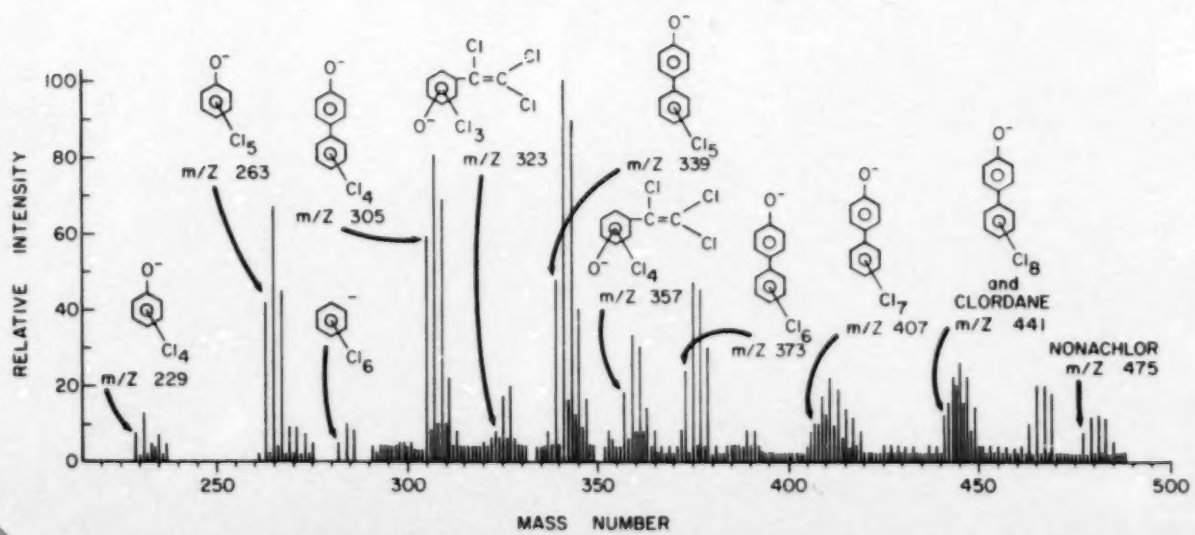

Figure 5. Negative chemical ionization mass spectrum of 50 mg of Lake Ontario lake trout cleaned up by GPC. Only ^{12}C negative mass defect are shown for polychlorinated chemicals

benzene, polychlorinated styrenes (*10*), chlordane, and nonachlor are readily identified in this sample (Figure 5).

Although the use of micromethods limits the possibilities for contamination of the sample, the amount of adventitious material that will appear as a positive is also smaller for microtechniques. Adequate procedural blanks must be carried through the entire procedure. We have used both water and corn oil for procedural blanks. Realistic tissue sample blanks would be desirable, but we are not aware of sources of sufficiently contaminant-free tissue. The steam distillation system may be the best for micromethods because smaller amounts of solvent and fewer pieces of glassware are required, so it is generally easier to obtain clean blanks with this system.

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RECEIVED for review October 19, 1979. Accepted January 28, 1980. This work was supported by grants from the National Institute of Health and the U.S. Environmental Protection Agency.